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A novel stability indicating HPLC-method for simultaneous determination of atenolol and nifedipine in presence of atenolol pharmacopeoial impurities

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ABSTRACT

For the first time a simple, rapid and accurate stability indicating HPLC method is described for simultaneous quantification of atenolol and nifedipine in bulk powder and dosage form. Chromatographic separation was carried out on Intersil® reversed phase C18 column. Separation was done using gradient binary mobile phase of ACN and 50 mM $NaClO_4$ in the ratio from 5: 95 to 50: 50 (v/v) within 8 minutes at flow rate of 1 mL/min and 30 °C. An UV detector was used at 230 nm for detection. The elution times of atenolol and nifedipine were found to be 6.05±0.02 and 14.50±0.04 minutes, respectively. The method was validated for system suitability, linearity, precision, limits of detection and quantitation, specificity, stability and robustness. Robustness study was done for small changes in temperature, flow rate, wavelength of detection and time to reach 50% of ACN in mobile phase. Stability tests were done through exposure of the analytes' solution for five different stress conditions. The limit of detection for both drugs was 0.04 µg mL^{-1}. Limits of quantitation were found to be 0.12 µg mL^{-1} for atenolol and 0.11µg mL^{-1} for nifedipine. The recovery value of this method was 100.40±0.85% for atenolol and 100.30±1.10% for nifedipine.

INTRODUCTION

Atenolol, 4-(2-hydroxy-3-isopropylamminopropoxy) phenylacetamide (Fig. 1), is a cardioselective beta blocker lacking intrinsic sympathomimetic activity. It is clinically used in the management of hypertension, angina pectoris, cardiac arrhythmias and myocardial infarction (Sweetman, 2006). Like other antihypertensive drugs, atenolol lowers the systolic and diastolic blood pressure by 15–20% in a single drug treatment and reduces cardiovascular mortality. It is also used alone or in combination with other antihypertensive agents for the treatment of myocardial infarction, arrhythmias, angina and disorders arising from decreased circulation and vascular constriction, including migraine (Prichard *et al.*, 2001). Nifedipine, 3, 5-dimethyl 2, 6-dimethyl-4-(2-nitrophenyl)-1, 4-dihydropyridine-3, 5-dicarboxylate (Fig. 1), is a dihydropyridine calcium-channel blocker. It is a peripheral and coronary vasodilator that has little or no effect on cardiac conduction and negative inotropic activity at therapeutic doses. Combination therapy of atenolol and nifedipine is now common and available since studies revealed that the combination regimen significantly reduced supine and standing systolic and diastolic blood pressure compared with each drug alone. Heart rate was significantly decreased by the combination compared with nifedipine alone (Stanley *et al.*, 1988).

In open literature, several methods have been reported for the determination of atenolol that relied on HPLC (Belal *et al.*, 2013; Bing *et al.*, 2004; Hui *et al.*, 2004; Vidyadhara *et al.*, 2012; Kallem *et al.*, 2013), gas chromatographic techniques (Yilmaz and Arslan, 2011), high performance thin layer chromatography (HPTLC) (Ramteke *et al.*, 2010), flourometry (Gajewska *et al.*, 1992), differential scanning calorimetry (DSC) and thermogravimetry (TG) (Pyramides *et al.*, 1995), electrophoresis (Azzam *et al.*, 2009), electrochemical methods (Taei *et al.*, 2015), atomic absorption spectrometry (AAS) (ElRies *et al.*, 1995), UV- and visible spectrophotometry (Prasad *et al.*, 1998; Singh *et al.*,

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1997; Ferraro *et al.,* 2003; Umapathi, 1994; Kasture, 2005; Veronico *et al.,* 1995; Sabel *et al.,* 2012) and titrimetry (Prashanth *et al.,* 2012). Atenolol was previously determined in combination with other beta-blockers (Yilmaz and Arslan, 2011), chlorthalidone (Azzam *et al.,* 2009; Ferraro *et al.,* 2003), amlodipine (Singh *et al.,* 1997) and hydrochlorothiazide as well as amiloride (Prasad *et al.,* 1998). HPLC method is being used most frequently for the trace analysis of nifedipine (Bing *et al.,* 2004; Hui *et al.,* 2004; Vidyadhara *et al.,* 2012; Kallem *et al.,* 2013; Asthana *et al.,* 2010; El Walily, 1997; Ohkubo *et al.,* 1992). Other instrumental techniques used for analysis of nifedipine are spectrophotometry (Umapathi, 1994; Kasture, 2005; Veronico *et al.,* 1995; Sabel *et al.,* 2012; El Walily, 1997; Shamsipur *et al.,* 2003), gas chromatography (El Walily, 1997; Martens *et al.,* 1994) and Spectrofluorometry (Al-Ghannam *et al.,* 2008). Electrochemical methods had been also described for determination of nifedipine (Shapovalov *et al.,* 2002; Squellaa *et al.,* 1989). Nifedipine was previously analyzed in combination with nateglinide and lovastatin (Asthana *et al.,* 2010), nicardipine and isradipine Martens *et al.,* 1994) as well as acebutolol HCl (El Walily, 1997).

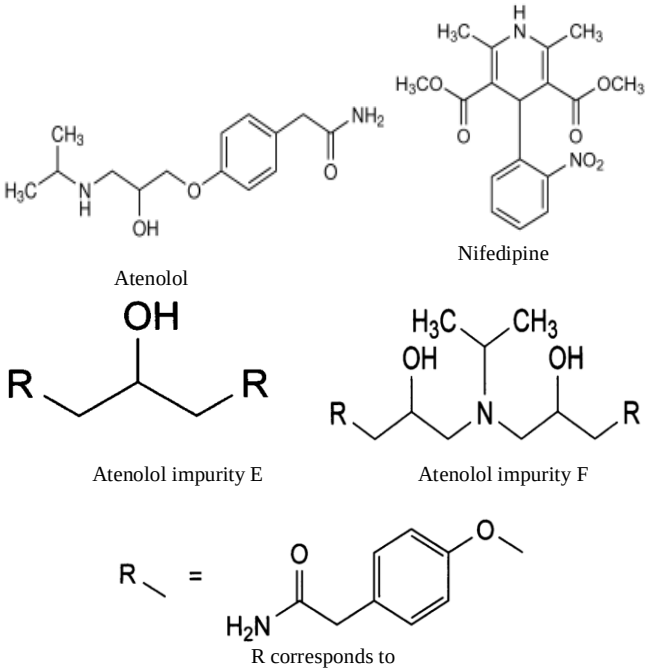


Fig. 1: Chemical structure of analytes and atenolol pharmacopeoial impurities.

Although there are various papers describing determination of each of atenolol and nifedipine alone or in combination with other drugs, only few papers described the determination of both drugs in combination by derivative spectrophotometry (Umapathi, 1994; Kasture, 2005; Veronico *et al.,* 1995; Sabel *et al.,* 2012), HPTLC (Ramteke *et al.,* 2010) and liquid chromatographic methods (Bing *et al.,* 2004; Hui *et al.,* 2004; Vidyadhara *et al.,* 2012; Kallem *et al.,* 2013). In the reported LC-methods (Bing *et al.,* 2004; Hui *et al.,* 2004; Vidyadhara *et al.,* 2012; Kallem *et al.,* 2013). For previously determination of both drugs in combination there are three limitations: Low sensitivity, use of ion pairing additive to facilitate the separation and these methods are not stability indicating methods. There is no a previous work dealt with stability indicating method for simultaneous determination of atenolol and nifedipine. In all reported stability indicating methods only one drug (atenolol or nifedipine) was determined. Handa *et al.,* (2014) found that presence of atenolol with nifedipine in the same dosage form led to accelerate degradation of nifedipine. Thus, it was important to find a new sensitive stability indicating method without using ion pairing agent for simultaneous quantitative determination of both drugs in combination.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents are at least analytical grade. Water was bidistilled, $NaClO_4$ was purchased from (Merck). ACN was HPLC-grade (J.T. Baker). Atenolol and nifedipine pharmaceutical grade were obtained from (EIPICo). Pharmaceutical formulation, Tenolate SR® capsules (containing 20 mg nifedipine and 50 mg atenolol per capsule) were obtained from Egyptian market.

Instrumentation

Agilent HPLC series 1200 (Agilent technologies) consists of solvent pump (model G1311A), autosampler (model G1329A), column compartment (model G1316A) and UV detector (model G1314A). SUNTEST CPS+® was used for UV-radiation for photodegradation.

Column

C18 stationary phase column Intersil® ODS-3 (5 µm, 4.6 x 150 mm) was obtained from GL sciences Inc..

Chromatography

The experiments were performed with gradiant elution. The binary mobile phase consisted of ACN and 0.05 M $NaClO_4$ (5: 95) at zero time to (50: 50) within the first 8 minutes then stayed 15 minutes 50: 50. The eluents were degassed before running, set at a flow rate of 1 $mLmin^{-1}$ and column temperature at 30 ºC. Volume of 20 µL of samples was injected per run and eluates were detected using UV -Detector at λ= 230 nm.

Solutions preparation

Preparation of stock and standard working solutions

The stock solutions of atenolol and nifedipine (1 mg mL^{-1}) were prepared by dissolving 100 mg of each in (1:1, v/v) ACN: H_2O to make 100 mL of solution. The standard working solutions were prepared by diluting aliquots of the stock solutions with (1:1, v/v) ACN: H_2O to obtain concentrations ranging from 2 to 50 µg mL^{-1}. The calibration graphs were constructed by plotting the peak areas obtained at wavelength 230 nm versus the corresponding injected concentrations.

Sample preparation

The contents of 10 capsules of Tenolate SR® were accurately weighed as fine powder. To an accurately weighed portion of the powder equivalent to one capsule, 250 mL (1:1) ACN: H_2O was added then the solution was left in the ultrasonic bad for 5 min. After that the solution was filtered and the first 10 mL was rejected then 5 mL of the filtrate was diluted to 100 mL using same solvent.

Stability tests

Forced degradation studies were performed to provide an indication of the stability-indicating properties and specificity of the method. Intentional degradation was attempted using acid, base, hydrogen peroxide, thermal and UV-radiation. A degradation sample was prepared by dissolving of 50 mg atenolol and nifedipine, each in 50 mL (ACN: H_2O, 1:1) through shaking and sonication. Then 10 mL of each solution was transferred into each of three 50 mL round bottom flasks to perform the first three degradation tests. To the first flask 10 mL of 1N HCl was added for acidic degradation. To the second flask 10 mL of 1N NaOH was added for basic degradation and to the third flask 10 mL of 30% H_2O_2 was added for oxidative degradation. Each of the three flasks was refluxed for about 4 hours.

After completing the degradation treatment, samples were allowed to cool to room temperature and treated as follows: The pH values of the first and second flasks were neutralized with 1N NaOH and 1N HCl, respectively. To the third flask 1N sodium bisulphite solution was added to destroy excess H_2O_2. The volume of all the three flasks was adjusted to 50 mL with (ACN: H_2O, 1:1). For thermal degradation, powders of atenolol and nifedipine were dispersed onto Petri-dish and left in oven at 60ºC for 4 hours then solution is prepared from them to concentration of 0.2 mg mL^{-1} using (ACN: H_2O, 1:1) as solvent. For degradation through UV-radiation 2 mL of the sample solution was left in UV radiation for 4 hours then the radiated solution diluted with (ACN: H_2O, 1:1) to 10 mL, then finally injected into LC and compared with control sample. Samples were injected and analyzed against control samples (lacking of degradation treatment).

The stock solutions of the specified impurities of atenolol in British Pharmacopoeia (containing atenolol impurity E and impurity F (The British Pharmacopoeia, 2011)) (Fig. 1) were prepared in concentration of 0.1 mg mL^{-1} (ACN: H_2O, 1:1) as solvent.

RESULTS AND DISCUSSION

Different types of RP-HPLC-columns were examined for separation of intact drugs from their stress degradants and from each other applying isocratic mode but no column of them enabled the baseline separation. Thus gradient mode was applied. Using of methanol as organic modifier resulted in elongation of retention times; so ACN was used as organic modifier. Mobile phase including water without any salt as aqueous part gave bad separation due to tailing of peaks and this is why $NaClO_4$ is added to aqueous part of the mobile phase. In previous studies, it was found that usage of $NaClO_4$ as aqueous mobile additive (chaotropic mobile phase additive) led to reduction of retention times and enhancement of separation of basic analytes via decreasing of tailing (Elhenawee *et al.*, 2014; Hashem *et al.*, 2014). The method was validated according to ICH guidelines (Guidance for Industry: ICH 1996) for system suitability, linearity, precision, limits of detection and quantitation, specificity, stability and robustness. Robustness study was done for small changes in temperature, flow rate, wavelength of detection and time to reach 50% of ACN in mobile phase.

System suitability

The results of three runs indicate high system suitability (table 1). The t_R-values of atenolol and nifedipine are 6.05±0.02 and 14.50±0.04 min, respectively. The RSD of peak areas are 0.60 and 0.90% for atenolol and nifedipine, respectively.

Table.1: System suitability, linearity and regression data for atenolol and nifedipine.

Parameters	Atenolol	Nifedipine
System suitability		
$t_R \pm$ SD (min)	6.05±0.02	14.50±0.04
N	6500	16000
k	3.9	9.5
Linearity range (µg mL^{-1})	2 - 25	2 - 25
Detection limit (µg mL^{-1})	0.04	0.04
Quantitation limit (µg mL^{-1})	0.12	0.11
Regression data		
Slope (b)	31.52	58.37
Intercept (a)	- 4.64	- 4.73
Coefficient of determination (R^2)	0.9999	0.9998

Linearity and Range

Six concentrations of atenolol and nifedipine solutions ranging from 2 to 50 µg mL^{-1} were analyzed. The graph of the peak area against concentration proved linearity in the range of 2 - 25 µg mL^{-1} and the linearity equation is: $Y = 31.518X - 4.64$ and coefficient of determination equals 0.9999 for atenolol, while for nifedipine the linearity equation is: $Y = 58.374X - 4.73$ and coefficient of determination equals 0.9998. The limit of detection (LOD) defined as the injected quantity giving S/N of 3.3 (in terms of peak height), was found to be 0.04 µg mL^{-1} for both atenolol and nifedipine. The limit of quantitation (LOQ) is defined as the injected quantity giving S/N of 10 (in terms of peak height), was found to be 0.12 µg mL^{-1} for atenolol and 0.11 µg mL^{-1} for nifedipine (table 1).

Accuracy and specificity of the method

The accuracy of the method was determined by recovery% using standard addition technique experiments (n=5). Atenolol and nifedipine showed high accuracy with recovery of 100.40±0.85 and 100.30±1.10%, respectively (table 2).

The comparison between the chromatogram of the raw atenolol or nifedipine (fig. 2a) and that of extracted from their dosage form (fig. 2b) indicates that the excipients in the formulation did not interfere with their determination. Also no

interference occurred from atenolol BP standard impurities (The British Pharmacopoeia, 2011) (fig. 2c). No interference was found from the following drugs: Amlodipine besylate, paracetamol, diazepam and hydrochlorothiazide, when they were simultaneously injected with atenolol and nifedipine.

Table 2: Accuracy of the proposed method applying standard addition technique (n=5).

Atenolol			Nifedipine		
Injected conc. (µg mL^{-1})	Calculated conc. (µg mL^{-1})	Recovery (%)*	Injected conc. (µg mL^{-1})	Calculated conc. (µg mL^{-1})	Recovery (%)*
3.48	3.48	100.00	3.24	3.26	100.52
6.27	6.29	100.26	4.30	4.32	100.59
7.77	7.83	100.76	8.59	8.54	99.37
10.43	10.38	99.48	10.74	10.66	99.24
16.69	16.97	107.73	17.18	17.52	101.90
Recovery % ± RSD					
100.40 ± 0.85%			100.30 ± 1.10%		

The results of stress degradation indicate that atenolol is more affected with reflux with NaOH (fig. 3a) and H_2O_2 (fig. 3b), while nifedipine is more affected with H_2O_2 (fig. 4a). Reflux with HCl (figs. 3c and 4b) led to degradation of atenolol and nifedipine but the effect here is weaker than in case of H_2O_2 and NaOH, while thermal (figs. 3d and 4c) and UV-light exposures (figs. 3e and 4d) gave the minimum effect on both atenolol and nifedipine. Although there are several degradants, there was no interference with the peaks of the intact drugs indicating that the method is stability indicating.

Stability of the analytical solution

Stability of the standard solution was studied by injection of the prepared solution at periodic intervals into the chromatograph up to about five days. The results indicate that the RSD of the peak area was within 1.00% for both atenolol and nifedipine.

Reproducibility and precision of the method

Results (table 3) show that there were high intra- and inter-day precisions (both within 2.00%). Intra-day precision was assessed through injection of the standard solution five times during a day at three concentrations. The same was done for inter-day precision test except that the injection of the samples was every day for five days.

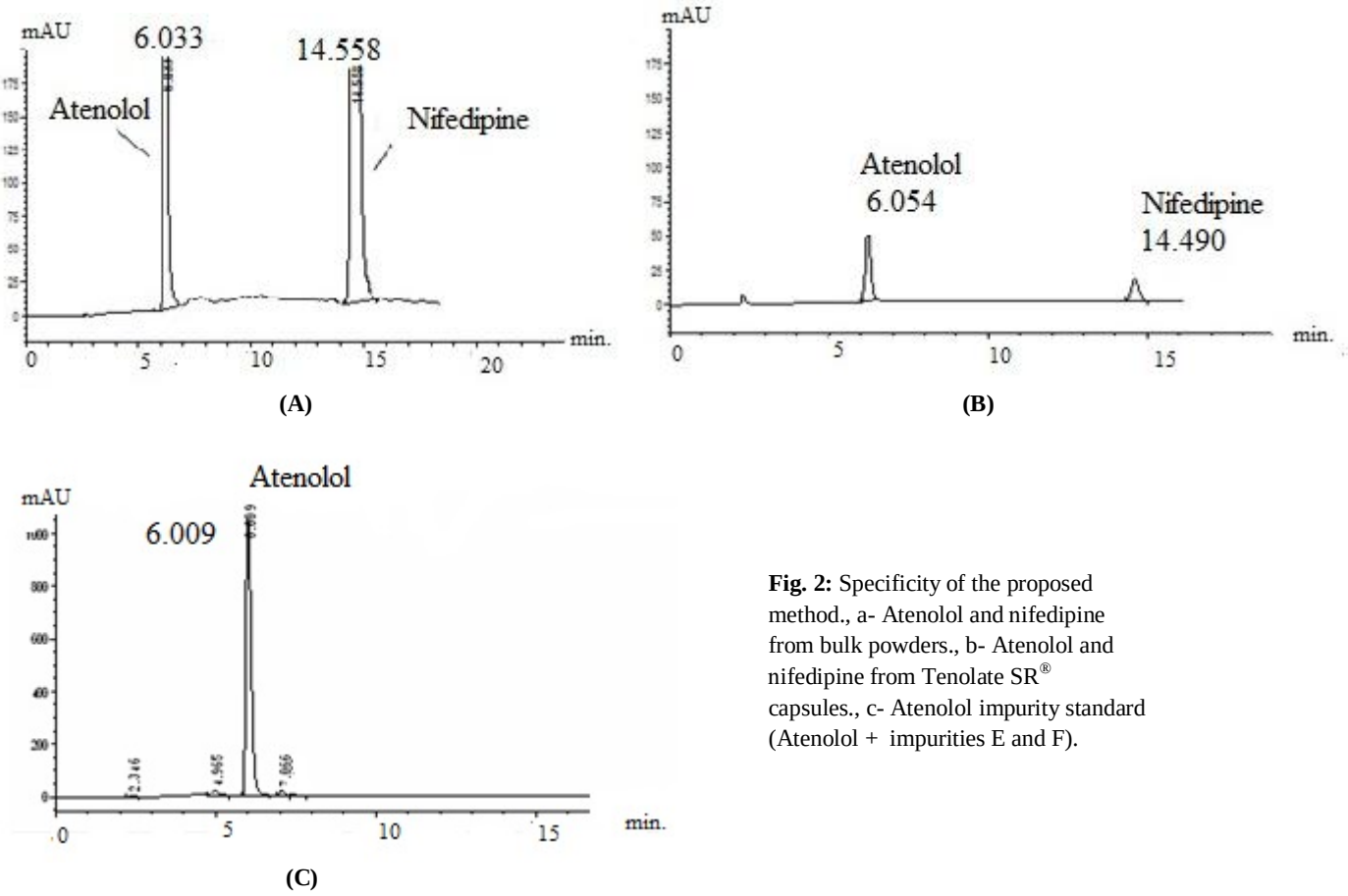
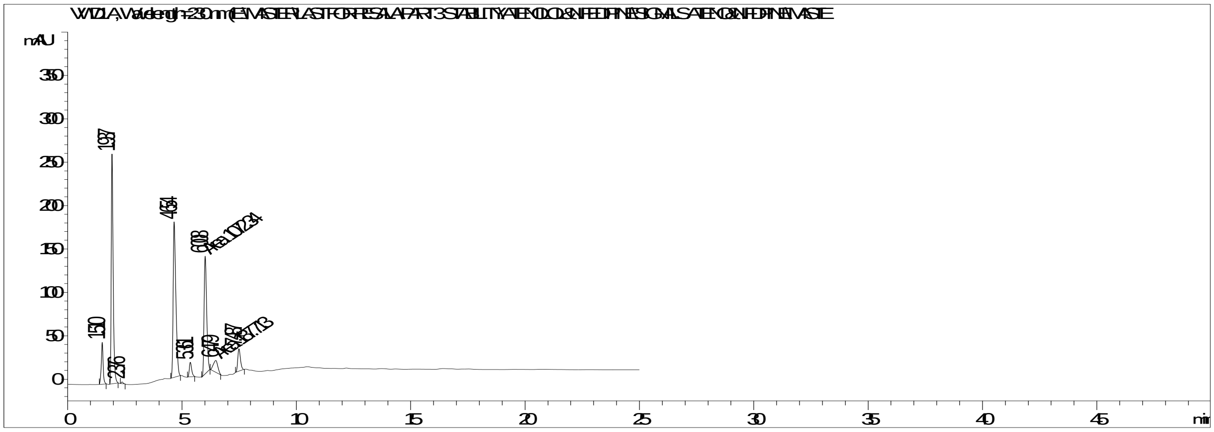


Fig. 2: Specificity of the proposed method., a- Atenolol and nifedipine from bulk powders., b- Atenolol and nifedipine from Tenolate SR® capsules., c- Atenolol impurity standard (Atenolol + impurities E and F).



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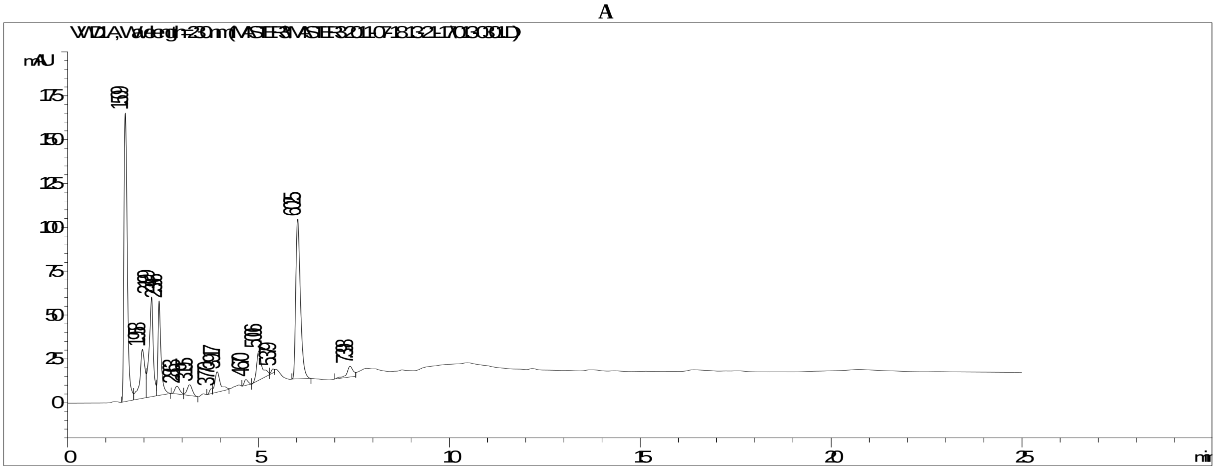

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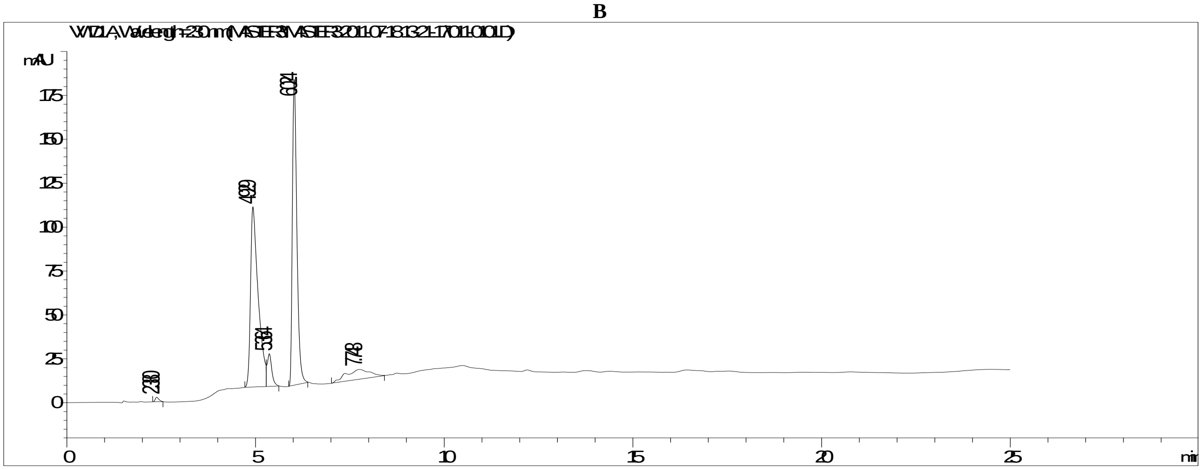

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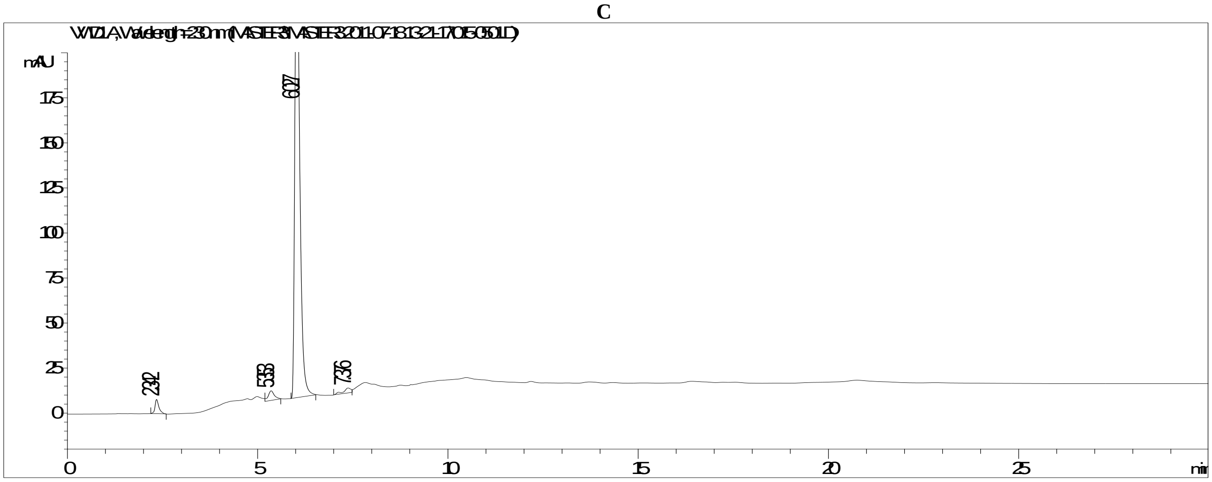

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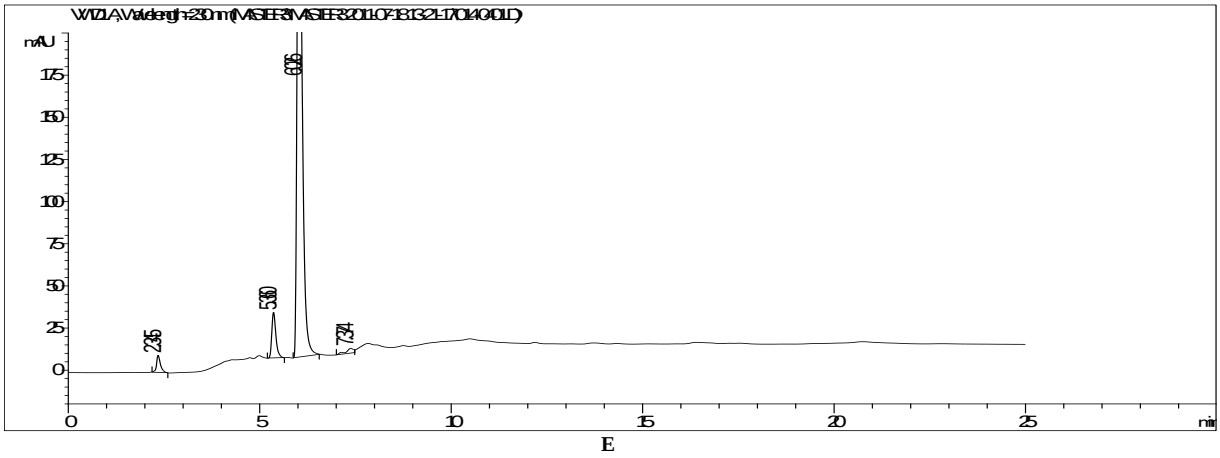

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Fig. 3: Stress degradation of Atenolol, A: Atenolol after treatment with 1N NaOH ., B: Atenolol after treatment with H_2O_2., C: Atenolol after treatment with 1N HCl., D: Atenolol powder after exposure to heat 60ºC for 4 hours., E: Atenolol after exposure to UV light at suntest® for 4 hours.

Table 3: Reproducibility and precision of the method.

Injected amount (µg mL^{-1})	Intra-day (n=5)			Inter-day (n=5)		
	Observed amount (µg mL^{-1})	CV %*	Accuracy (%)**	Observed amount (µg mL^{-1})	CV %*	Accuracy (%)**
5.00	4.94	0.33	98.84	4.99	0.90	99.70
10.00	9.99	0.10	99.89	10.07	0.85	100.70
20.00	20.15	0.04	100.70	20.30	0.70	101.50
			Atenolol			
10.08	10.08	0.68	100.00	10.08	0.50	100.00
20.16	20.37	0.89	101.00	20.26	0.50	100.50
25.20	25.04	0.74	99.40	24.96	0.38	99.04
			Nifedipine			

*Coefficient of variation (%) = S.D. /mean x 100., **Accuracy (%) = observed concentration /used concentration x 100.

Table 4: Statistical comparison of the proposed method with a reported method (Vidyadhara et al., 2012)

Drug	Atenolol		Nifedipine	
	Proposed method	Reported method (Vidyadhara et al., 2012)	Proposed method	Reported method (Vidyadhara et al., 2012)
Mean ± SD	100.78±0.98	100.18±0.76	100.65±1.02	100.19± 0.92
N	6	6	6	6
RSD	0.972	0.759	1.013	0.918
V	0.960	0.578	1.040	0.846
Student-t	1.185 (2.20)*		0.821 (2.20)*	
F-test	1.660 (4.12)*		1.229 (4.12)*	

* The figures in parenthesis are the theoretical values for t- and f-test at (P = 0.05).

Table 5: Robustness of the proposed method.

Slight changes in							
Temp. (C°) (28, 30 and 32°C)		**Flow rate (mL min^{-1}) (0.9, 1.0 and 1.1)**	**Time to reach 50% ACN (minutes) (7.8, 8.0 and 8.2)**		**wavelength of detection (nm) (228, 230 and 232)**	**$NaClO_4$ conc. (mM) (40.0,50.0 and 60.0)**	
CV (%)* of affected factor							
Peak area	t_R	K´	Peak area	t_R	t_R	Peak area	K´
Atenolol							
0.20	0.50	3.20	1.90	0.84	0.09	1.86	0.67
Nifedipine							
0.70	0.70	4.40	0.38	1.09	0.03	0.81	0.27

*Coefficient of variation (%) = S.D. /mean x 100.

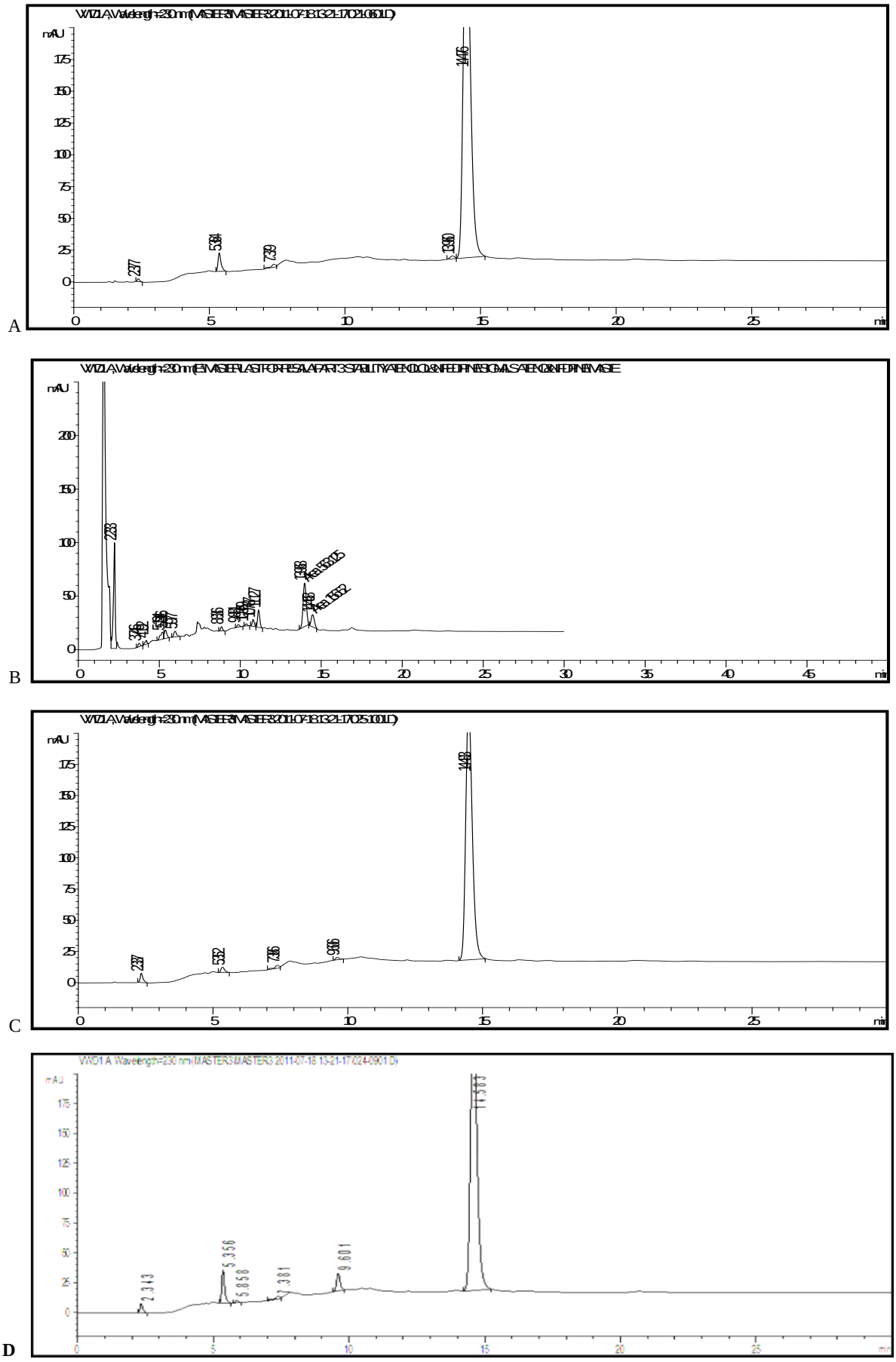

Fig. 4: Stress degradation of Nifedipin., a: Nifedipine after treatment with H_2O_2., b: Nifedipine after treatment with 1N HCl., c: Nifedipine powder after exposure to heat 60ºC for 4 hours., d: Nifedipine after exposure to UV light at suntest® for 4 hours

Application

The analysis of atenolol and nifedipine in Tenolate SR® (table 4) capsules showed high accuracy with recovery of 100.78±0.98 and 100.65±1.013%, respectively. The results were compared with a reported method (Vidyadhara *et al.*, 2012) using t- and F-values and there was no significant difference.

Robustness of the method

The robustness of the present method was evaluated in the terms of temperature, flow rate, time to reach 50% ACN in mobile phase, wavelength of detection, salt concentration and injection volume (table 5). The slight variations in the examined factors had no significant effect on the shape of the peak. The results indicate that the method is more sensitive to changes in flow rate.

CONCLUSIONS

A valid and fast stability indicating HPLC-method for simultaneous quantification of atenolol and nifedipine is established. Compared with the published methods this method represents a good reduction of the time. With the proposed method a satisfactory separation of atenolol and nifedipine both from each other and from the degradation products and pharmacopeoial impurities was achieved. Extended linear range and rapid analysis time was carried out. A high recovery of both drugs in formulation was achieved. The proposed method ensured a precise and accurate determination of atenolol and nifedipine in oral capsules formulation and is stability indicating method. No interference from the excipients was noticed.

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There is no any conflict of interest.

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